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Sequence Analysis of NSP4 Gene of Human Rotavirus Allows Classification into Two Main Genetic Groups

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The rotavirus nonstructural glycoprotein NSP4 may represent the first identified viral enterotoxin. We have sequenced reverse transcription-polymerase chain reaction (RT-PCR)-generated fragments of 16 NSP4 genes of human rotavirus (HRV) strains from six different countries, representing seven different G and P type combinations. Based on the amount of sequence divergence between these and 11 previously sequenced NSP4 genes of human and animal rotaviruses, three distinct genetic groups could be recognized. Most strains within a group were closely related to each other at the nucleotide (nt) and amino acid (aa) levels (usually <10% divergence) but more distantly related (maximum 30.0% nt divergence and 24.7% aa divergence) to members of the other groups. Intergroup variation occurred in two highly variable regions of NSP4 (aa 16–34 and aa 131–148). The NSP4 ''toxic peptide'' (aa 114–135) exhibited aa variation at its carboxy terminus both within and between genetic groups. The largest group (genetic group II) contained HRV strains of subgroup II specificity (including genotypes P[8]G1, P[8]G3, P[6]G3, and P[8]G5 and serotype P8[11]G9), and the smaller group (genetic group I) contained HRV strains of subgroup I specificity (genotype P[4]G2). The NSP4 sequence of the rhesus rotavirus vaccine strain was distinct from all other strains and formed the third group (genetic group III). The NSP4 genes of animal rotaviruses UK, NCDV, and SA11 (genetic group I) and YM (genetic group II) and two possible human-animal rotavirus reassortant strains, Brazilian P[8]G5 and Indian P[11]G9 (genetic group II), could also be classified into one of these groups, suggesting a close evolutionary relationship between human and animal NSP4 genes. These results will facilitate studies of the host immune response to NSP4, which may be relevant to future HRV vaccine design. ***J. Med. Virol. 53:41–50, 1997.*** © 1997 Wiley-Liss, Inc.†

KEY WORDS: HRV; NSP4; toxic peptide; genotypes

INTRODUCTION

Group A rotaviruses are the leading cause of severe gastroenteritis in infants and young children worldwide, and the development of a rotavirus vaccine has thus been assigned a high priority [Institute of Medicine, 1986]. Although much progress has been made toward the goal of a safe and effective vaccine [Kapikian et al., 1996], our understanding of the basic mechanisms of rotavirus pathogenicity and of immunity to rotavirus infections remains limited. Rotaviruses are nonenveloped RNA viruses that possess 11 segments of double-stranded RNA, enclosed within a triple-layered protein capsid. Each genome segment encodes a viral protein (VP), including six structural proteins and five nonstructural proteins [Estes, 1996b].

The nonstructural transmembrane glycoprotein NSP4, the product of genome segment 10, is one of several rotavirus genes (including those coding for

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VP3, VP4, VP7, NSP1, and NSP2) that has been associated with pathogenicity in different hosts, but no single gene has been shown universally to determine rotavirus pathogenicity [Hoshino et al., 1995; Burke and Desselberger, 1996; Greenberg et al., 1994]. NSP4 is known to play a role in virus assembly [Estes, 1996b]. More recently, NSP4 and, specifically, a 22-amino-acid (aa) synthetic peptide corresponding to NSP4 residues 114–135, has been shown to induce diarrhea in young mice and may be a key determinant of rotavirus pathogenicity [Ball et al., 1996; Tian et al., 1994, 1995]. This response was age and dose dependent and specific. It is postulated that NSP4 acts as a viral enterotoxin and causes diarrhea by triggering a signal transduction pathway in intestinal epithelial cells that results in an increase in intracellular [Ca^+], thus stimulating cAMP-dependent Cl^- secretion through a Ca^+-dependent signaling pathway. This mechanism was compared to mechanisms used by bacterial enterotoxins and the endogenous peptide guanylin [Ball et al., 1996]. Tyrosine at position 131 was identified as a crucial aa required for activity of the NSP4 "toxic peptide"; a mutant peptide in which lysine was substituted for tyrosine at this position did not induce diarrhea [Ball et al., 1996]. It has further been shown that pups born to dams immunized with the NSP4 114–135 peptide, when challenged with a high dose of infectious simian rotavirus SA11, developed rotavirus diarrhea of reduced frequency and severity. In addition, the oral administration of NSP4-specific antibody to young pups infected with SA11 significantly reduced subsequent diarrheal disease [Ball et al., 1996].

A second line of evidence supporting a role for NSP4 in the pathogenesis of diarrhea has come from studies of a cell-culture-attenuated variant of the porcine rotavirus strain OSU [Zhang et al., 1996]. It was demonstrated that loss of the virulent phenotype (i.e., induction of diarrhea in gnotobiotic piglets) in the attenuated strain was accompanied by aa substitutions in the NSP4 protein between residues 131 and 140, representing the carboxy-terminal one-fourth of the toxic peptide, and five additional aa residues. Furthermore, it was subsequently shown that the baculovirus-expressed NSP4 protein from the attenuated OSU variant had a reduced ability to induce diarrhea in young mice, whereas the NSP4 protein of the virulent parent virus was able to induce diarrhea [Zhang et al., 1996].

These studies suggest a potential role for NSP4 in the mechanism of rotavirus diarrhea in infants. Thus, investigations in this area might lead to an improved understanding of rotavirus pathogenicity. Moreover, the finding that antibodies to NSP4 can reduce the ability of this protein to cause diarrhea in a mouse model suggests that it will be important to determine whether the development of antibodies to NSP4 in infants after natural infection or vaccination correlates with subsequent protection from rotavirus disease [Ball et al., 1996; Estes, 1996a]. Such studies could lead to alternative vaccination strategies to control rotavirus diarrhea [Glass et al., 1996]. Knowledge of NSP4 diversity in nature might also provide information regarding rotavirus evolution. Among common rotavirus serotypes of infants, only the strain Wa NSP4 gene sequence has previously been reported [Okada et al., 1984]. The few other published sequences are from strains isolated from human neonates, two strains isolated from a chronically infected immunodeficient child and the animal rotaviruses UK, NCDV, YM, and SA11 [Powell et al., 1988; Kirkwood et al., 1996; Baybutt and McCrae, 1984; Ballard et al., 1992; Both et al., 1983; Lopez and Arias, 1993; Ward et al., 1985].

We have examined the degree of genetic diversity among NSP4 genes of human rotavirus (HRV) strains of diverse origin and various G and P genotypes. We hypothesized that, if NSP4 is a rotavirus enterotoxin, then the protein, or at least the toxic peptide region, might be conserved between HRV strains of different genotypes from diverse geographic locations. We also wished to assess the extent of heterogeneity between circulating strains and vaccine candidates. Our results suggest that two main groups of NSP4 genes are present among HRVs and that these groups are highly divergent from the NSP4 gene of the rhesus rotavirus (RRV) vaccine strain, which represents a third genetic group.

MATERIALS AND METHODS

Rotavirus Strains

The origins of most of the fecal rotavirus strains and culture-adapted viruses used in this study have been previously described [Gentsch et al., 1992; Bhan et al., 1993; Leite et al., 1996]. Strains from the United States were isolated during a recent rotavirus vaccine trial [Rennels et al., 1996], culture-adapted (R.L. Ward, unpublished data, 1996), and G serotyped and P genotyped by standard methods [Taniguchi et al., 1987; Gentsch et al., 1992]. Strains from Thailand [Hendricks et al., 1995], the Philippines [Paje-Villar et al., 1994], and Uganda (C.A. Hart, unpublished data, 1996) were collected during epidemiologic studies of pediatric gastroenteritis. Strains 116E and Ug71 were detected in asymptomatic neonates. The remainder of the strains were recovered from diarrheic infants (Table I). All stools were stored frozen at −80°C. Ten percent suspensions were made in phosphate-buffered saline and were tested for the presence of group A rotavirus antigen by using a commercially available enzyme-linked immunosorbent assay (ELISA; Rotaclone, Cambridge Biotech, Worcester, MA). Subgroup determination was performed using an ELISA with subgroup-specific monoclonal antibodies applied to fecal suspensions or viral cell lysate [Greenberg et al., 1983]. Genomic double-stranded RNA was extracted using a glass powder method [Gentsch et al., 1992]. Rotavirus G and P genotypes were determined using a one-step reverse transcription-polymerase chain reaction (RT-PCR) procedure that we have described previously [Gouvea et al., 1990; Gentsch et al., 1992; Das et al., 1994].

TABLE I. HRV Strains, Subgroups, P and G Genotypes, Country of Origin, and Year Isolated

Strain	Subgroup	P and G type[a]	Country	Year isolated	Reference
Field isolates					
GIMR7[b]	II	P[8]G1	United States	1992	R.L. Ward, unpublished data, 1996
UL274[b]	II	P[8]G1	United States	1992	R.L. Ward, unpublished data, 1996
CDC125[b]	II	P[8]G1	United States	1992	R.L. Ward, unpublished data, 1996
RIH14[b]	II	P[8]G1	United States	1992	R.L. Ward, unpublished data, 1996
Br1067	II	P[8]G5	Brazil	1984–92	Leite et al., unpublished data, 1996
Ug70	II	P[8]G4	Uganda	1994	C.A. Hart, unpublished data, 1996
Ug71[c]	II	P[6]G3	Uganda	1995	C.A. Hart, unpublished data, 1996
Ug72	II	P[6]G3	Uganda	1995	C.A. Hart, unpublished data, 1996
116E[c]	II	P[11]G9	India	1987	Bhan et al., 1993
H120	NG[d]	P[4]G2	India	1993	Ramachandran et al., 1996
H150	NG[d]	P[8]G1	India	1993	Ramachandran et al., 1996
Th36	II	P[8]G3	Thailand	1990	Hendricks et al., 1995
Th41	II	P[8]G3	Thailand	1990	Hendricks et al., 1995
Th44	I	P[4]G2	Thailand	1990	Hendricks et al., 1995
Ph49	I	P[4]G2	Philippines	1990	Paje-Villar et al., 1994
Ph52	ND[e]	P[4]G2	Philippines	1990	Paje-Villar et al., 1994
Reference strains					
Wa	II	P1A[8]G1	United States	1979	Wyatt et al., 1983
RV4	II	P1A[8]G1	Australia	1977	Albert and Bishop, 1984
RV5	I	P1B[4]G2	Australia	1977	Albert and Bishop, 1984
S2	I	P1B[4]G2	Japan	1982	Urasawa et al., 1982
M37	II	P2A[6]G1	Venezuela	1982	Wyatt et al., 1983
A28	I	P[?[f]]G10	United Kingdom	1987	Hundley et al., 1987
SA11	I	P[2]G3	South Africa	1967	Malherbe et al., 1967
RRV	I	P5[3]G3	United States	1980	Stuker et al., 1980
NCDV	I	P6[1]G6	United States	1971	Mebus et al., 1971
UK	I	P7[5]G6	United Kingdom	1978	Woode, 1978
YM	I	P9[7]G11	Mexico	1983	Ruiz et al., 1989

[a]Rotavirus designations as recommended by the Rotavirus Nomenclature Working Group [Estes, 1996b].
[b]Strains GIMR7, UL274, CDC125, and RIH 14 are "vaccine failure strains" obtained during a recent U.S. rotavirus vaccine trial from vaccine recipients with rotavirus diarrhoea [Rennels et al., 1996].
[c]Strains 116E and Ug71 were detected in asymptomatic neonates.
[d]Strains H120 and H150 did not subgroup.
[e]Ph52 subgrouping not done (insufficient sample).
[f]The P type has not been reported.

RT-PCR Amplification of NSP4 Gene

RNA extracted by the glass powder method was used for RT-PCR amplification of fragments of the NSP4 gene, using the primers described in Table II. The following PCR conditions were used: one cycle at 94°C for 3 min; 40 cycles each at 94°C for 1 min, 51°C for 2 min, and 72°C for 3 min; and one cycle at 72°C for 7 min. For nucleic acid sequencing, the ethidium bromide-stained products were extracted and purified from a 1.2% SeaKem GTG agarose gel (FMC Bioproducts, Rockland, ME) using the QIAquick Gel Extraction Kit procedure (Qiagen Inc., Chatsworth, CA).

Cycle Sequencing

DNA sequencing was performed by the dideoxynucleotide chain termination method, using the PRISM TM Ready Reaction DyeDeoxy TM Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA) with the thermal cycling conditions recommended by the manufacturer. The oligonucleotide primers used for cycle sequencing are described in Table II. The products were purified on Centrisep columns (Princeton Separations, Adelphia, NJ) and sequenced using a model 377 Automated DNA sequencer (Applied Biosystems, Inc.).

To generate double-stranded DNA templates for cycle sequencing, one or two RT-PCR products were prepared for each strain, using complementary consensus primers located near the 5′ and 3′ ends of the gene that would amplify both genetic group I and genetic group II NSP4 genes (see footnote to Table II). These primers and a series of internal primers were then used for cycle sequencing reactions. The minimum sequence obtained for all strains was located between nucleotides (nt) 66 and 551 of the rotavirus NSP4 gene (aa 9 and 170 of the NSP4 protein). Overlaps in sequence between the different products were analyzed with the Sequencher program (Gene Codes Corporation, Inc., Ann Arbor, MI). Comparison to other rotavirus sequences was performed using the University of Wisconsin Genetics Computer Group and the Phylip 3.5 computer programs [Devereux et al., 1984; Felsenstein, 1989].

The new HRV gene 10 sequences appearing in this paper have been deposited in the GenBank sequence database and given the following accession numbers: U78558 (116E), U78559 (Br1067), U78560 (CDC125), U78561 (GIMR7), U78562 (HI20), U78563 (H150), U78564 (Ph49), U78565 (Ph52), U78566 (RIH14), U78567 (Th36), U78568 (Th41), U78569 (Th44),

TABLE II. RT-PCR and Sequencing Primers

Primer sequence (5′–3′)	HRV strain	Nucleotide position	Polarity
taa aag ttc tgt tcc gag ag[a]	Wa	7–26	+
gat tgg tta aac ggg att a[a]	Wa	687–705	−
tct ttt aaa att ggc tgg ata	116E	275–95	+
acg gca act caa cct ct	116E	569–85	−
aag cgt caa ttc caa caa tg	H120	184–203	+
ttt ccg ctc tcc cac tct	H120	512–29	−
gac gtc agc tgg aga tga	116E	380–97	+
cca gct gac gtc tca tct c	116E	373–91	−
ggc ttt taa aag ttc tgt t	Wa	1–19	+
acc att cct tcc att aac	Wa	721–38	−

[a]These primers were used to generate most of the PCR products and were also used for cycle sequencing; the remaining primers were used mainly for cycle sequencing.

U78570 (Ug70), U78571 (Ug71), U78572 (Ug72), and U78573 (μl274).

RESULTS

We investigated the genetic diversity of rotavirus NSP4 genes by analysis of 11 human and animal rotavirus NSP4 gene sequences available from published reports or from the database and by sequence analysis of NSP4 gene fragments of 16 HRV strains from six countries, representing seven different G and P type combinations. The HRV strains examined in the present study included both globally common childhood diarrhea strains, such as genotypes P[8]G1 (United States, India), P[8]G4 (Uganda), P[8]G3 (Thailand), and P[4]G2 (India, Thailand, the Philippines), as well as globally less common strains that are prevalent in particular countries, such as genotype P[8]G5 (Brazil), and two strains from asymptomatic neonates, genotypes P[9]G11 (India), and P[6]G3 (Uganda). These strains were compared to the NSP4 genes of previously characterized reference strains (Table I). The region of the gene sequenced represented approximately 90% of the open reading frame (ORF) of the 175-amino-acid NSP4 glycoprotein (i.e., nt 66–551, aa 9–170). The nucleotide sequence corresponding to the entire 175 aa NSP4 protein of several strains has been completed, and this has resulted in no significant differences in the conclusions reached through sequencing only 90% of the ORF [Cunliffe et al., 1997] (M. Ramachandran, M.K. Bhan, R.I. Glass, J.R. Gentsch, unpublished data, 1996).

The nt and aa sequence divergence of NSP4 genes between these strains and eight reference strains ranged from 0.0% to 30.0% (nt) and from 0.0% to 24.7% (aa; Table III). However, three distinct groups were discernible based on the calculated divergence values between NSP4 genes. The largest group (designated genetic group II) consisted of strains whose NSP4 genes were closely related (i.e., usually <10% aa or nt divergence for most strains) to each other and to typical HRV subgroup II strains, such as Wa and M37, but were distantly related to typical subgroup I strains, such as RV-5. The second, smaller group of strains (designated genetic group I) was closely related to subgroup I strain RV-5 (aa or nt divergence of 5–14% for most strains) but highly divergent from the larger group. Strain RRV NSP4 represents a third genetic group (genetic group III).

Several exceptions to these patterns were found. For example, the NSP4 gene of porcine strain YM was closely related (i.e., <5.1% aa divergence) to Wa and many other strains in genetic group II (e.g., Ug70, Br1067, 116E) sequenced in this study. Furthermore, in genetic group I, two animal rotaviruses analyzed, UK [Baybutt and McCrae, 1984; Ward et al., 1985] and SA11 [Both et al., 1983], were highly homologous to RV-5 and several strains characterized here (e.g., Ph49, Ph52, Th44; <9.2% aa divergence).

The patterns of aa substitutions between groups were analyzed using an aa alignment, with strain Wa as the reference sequence (Fig. 1) [Okada et al., 1984]. Most aa substitutions occurred near the amino terminus of the sequenced region (aa 16–34) and at aa 131–148, with smaller numbers of scattered substitutions throughout the rest of the protein fragment. Within these two regions, the NSP4 peptides contained numerous aa sequence differences that were common to most members of the same genetic group (e.g., Leu-25, Glu-26, Tyr-131, Lys-133, Ile-148 of genetic group I) but were not found in most other strains. Several other residues that differed between groups (e.g., residues 19, 76, 141, 145) were not completely conserved within genetic group I. Strain RRV, and to a much lesser extent other strains, such as Ug71 and Ug72, appeared to have distinctive patterns of aa substitutions. Of note was that aa 114–130 of the toxic peptide were highly conserved between genetic groups, whereas three of five aa at the carboxy end of this peptide were divergent between groups.

Phylogenetic analysis of these patterns of aa substitutions confirmed that rotavirus NSP4 proteins belong to at least three distinct genetic groups represented by prototype strains RV-5 (genetic group I), Wa (genetic group II), and RRV (genetic group III; Fig. 2). In addition, it is evident that some divergence within genetic groups I and II has occurred. This analysis confirms the evolutionary relation between the NSP4 proteins of typical subgroup I rotaviruses and animal rotaviruses,

TABLE III. Nucleotide (nt 66–552) and Amino Acid (aa 9–170) Divergence Between NSP4 Gene Regions of Rotaviruses[a]

	nt divergence[b]																					
Strain	Ug70	Br1067	116E	GIMR7	Wa	UL274	M37	H150	Th36	Th41	CDC125	RIH14	Ug71	YM	SA11	Ph49	Ph52	H120	Th44	A28	RV-5	UK	RRV
Ug70	—	2.7	7.3	7.3	4.0	4.7	6.4	7.3	5.8	5.8	4.0	4.9	2.5	13.2	23.8	22.7	22.4	21.5	21.5	24.9	21.8	22.9	23.2
Br1067	2.5	—	7.1	6.4	3.8	4.5	6.2	7.1	5.1	5.1	3.8	4.5	4.0	12.3	24.6	22.4	23.6	20.7	22.4	24.1	21.8	22.9	21.5
116E	5.8	7.1	—	0.4	5.3	6.4	6.4	9.9	6.4	6.4	5.8	6.7	8.5	10.4	24.3	21.8	21.5	20.2	20.7	22.7	21.5	21.8	23.5
GIMR7	5.1	6.5	0.6	—	5.3	6.4	6.4	9.2	6.4	6.4	5.8	6.4	8.5	10.4	24.6	21.8	23.0	20.2	21.0	22.9	21.8	21.8	23.2
Wa	3.1	4.5	5.1	4.5	—	2.3	4.5	6.0	4.2	4.2	1.7	2.3	5.8	12.3	24.9	23.2	24.5	22.1	23.5	24.1	23.2	22.4	24.1
UL274	3.1	4.5	6.5	5.8	3.2	—	4.7	6.4	5.3	5.3	1.5	1.2	6.4	13.0	25.8	24.1	25.1	22.9	24.1	24.9	23.8	23.2	24.3
M37	5.8	7.1	7.8	7.1	3.2	5.1	—	8.7	7.6	7.6	5.3	5.6	7.8	13.5	23.8	22.4	23.6	20.7	21.5	24.3	22.4	22.7	24.6
H150	5.1	6.5	7.8	7.1	5.1	5.8	7.8	—	7.8	7.8	6.2	6.0	9.2	14.0	24.9	22.7	23.3	22.1	23.2	24.1	22.7	21.8	22.1
Th36	3.2	5.8	6.5	5.8	5.1	6.5	7.8	7.8	—	0.0	4.7	5.3	7.1	12.5	24.3	22.9	23.9	21.8	22.7	24.3	22.9	22.1	24.1
Th41	3.2	5.8	6.5	5.8	5.1	6.5	7.8	7.8	0.0	—	4.7	5.3	7.1	12.5	24.3	22.9	23.9	21.8	22.7	24.3	22.9	22.1	24.1
CDC125	3.2	9.2	6.5	6.5	3.8	2.5	6.5	5.8	5.8	5.8	—	4.0	5.8	15.2	26.4	23.5	24.5	21.5	24.9	25.8	24.6	24.6	27.5
RIH14	4.5	5.8	7.8	7.1	5.1	2.5	7.8	5.8	7.8	7.8	3.8	—	6.7	13.5	25.2	24.1	25.1	22.9	24.1	24.9	23.8	23.2	24.6
Ug71	4.5	4.5	9.2	8.5	6.5	6.5	8.5	8.5	7.8	7.8	6.5	14.3	—	14.2	25.2	23.2	22.9	22.1	21.8	25.8	22.4	23.5	23.8
YM	5.8	7.1	5.8	5.1	5.1	5.8	7.8	7.8	7.8	7.8	5.8	7.1	9.2	—	25.2	26.1	26.9	25.5	25.8	30.0	26.4	27.2	27.2
SA11	19.7	20.5	20.5	19.7	20.5	21.3	19.7	18.9	20.5	20.5	21.3	21.3	21.3	19.7	—	16.3	16.0	15.7	16.5	16.5	15.0	13.7	29.0
Ph49	18.1	18.9	18.1	17.3	18.1	18.1	18.1	18.1	18.9	18.9	17.3	19.7	18.9	18.9	7.8	—	0.7	5.1	8.0	14.5	5.3	8.5	27.2
Ph52	17.6	19.3	17.3	17.7	17.7	18.5	17.7	18.5	18.5	18.5	16.6	20.2	18.1	19.3	7.6	0.0	—	5.1	8.2	14.2	5.9	8.7	28.2
H120	15.8	16.6	15.8	15.0	15.8	16.6	15.8	15.8	17.3	17.3	15.8	18.1	16.6	17.3	6.5	3.2	2.5	—	5.6	13.7	6.4	9.0	26.1
Th44	18.9	19.7	19.7	18.9	19.7	20.5	19.7	20.5	21.3	21.3	19.7	22.2	19.7	21.3	9.2	7.8	7.6	5.1	—	14.9	7.3	10.4	28.4
A28	18.1	18.9	16.6	15.7	18.1	18.9	18.1	18.9	19.7	19.7	18.1	20.5	19.7	18.1	9.2	6.9	6.9	5.1	9.2	—	14.2	14.5	24.3
RV-5	19.7	18.1	19.7	18.9	19.7	20.5	19.7	19.7	20.5	20.5	19.7	22.2	20.5	20.5	7.1	5.8	5.4	5.1	9.2	7.8	—	9.2	24.9
UK	17.3	20.5	17.3	16.5	17.3	18.1	17.3	17.3	18.1	18.1	17.3	19.7	18.1	18.1	2.6	3.2	2.6	2.5	6.5	5.1	2.5	—	28.4
RRV	17.3	15.7	18.9	18.1	17.3	18.1	18.9	18.9	18.1	18.1	17.3	19.7	24.7	17.3	22.1	20.5	20.2	18.1	21.3	18.9	19.7	18.9	—
	aa divergence[b]																						

[a]Eight reference sequences were used in the analyses with strains sequenced in this study: Wa (Okada et al., 1984; accession No. K02032); UK (Baybutt and McCrae, 1984; Ward et al., 1985; K03384); SA11 (Both et al., 1983; K01138); YM (Lopez and Arias, 1993; X69485); RRV (Tang and Greenberg, unpublished data, 1996; L41247); A28 (Ballard et al., 1992; D01145); RV-5 (Palombo and Kirkwood, unpublished data, 1996; U59103); M37 (Palombo and Kirkwood, unpublished data, 1996; U59109).
[b]The boxed areas represent strains that fall within genetic groups I (smaller box) and II (larger box).

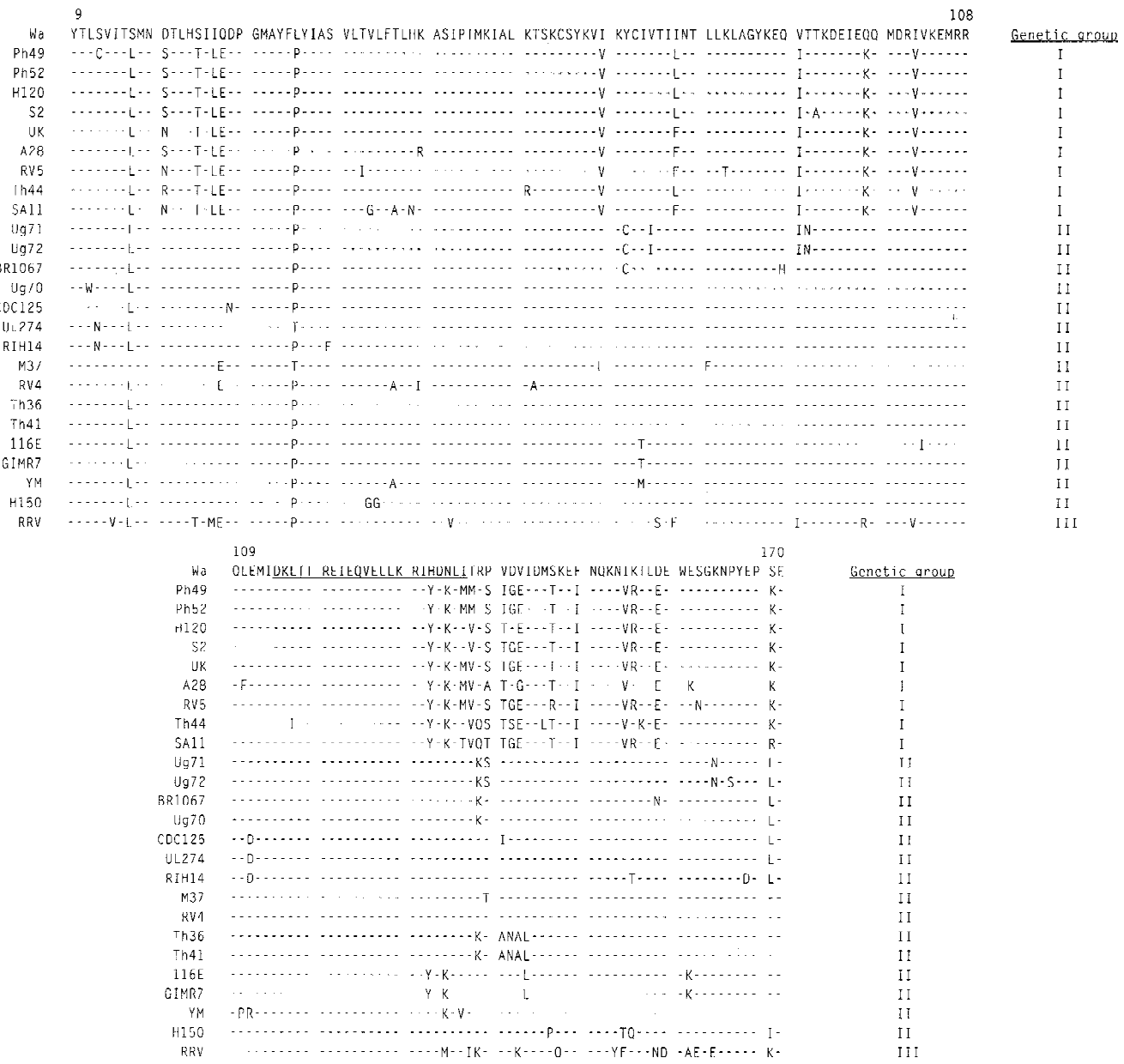

Fig. 1. Comparison of deduced amino acid (aa) sequences (residues 9–170) of the NSP4 peptides of 26 human and animal rotaviruses. Sequence alignments were prepared by the GCG program PILEUP, using a progressive alignment method [Devereux et al., 1984]. The sequence of strain Wa (top line) was used as the reference, and the aa that differ from Wa are shown for the remaining strains, using the single-letter aa code. The proposed toxic peptide region (aa 114–135) is underscored. Several strains included in Figures 1 and 2 (Ug72, S2, RV4, and NCDV) were not included in Table III. The sequences for S2 (E.A. Palombo and C.D. Kirkwood, unpublished data, 1996; U59104), RV4 (E.A. Palombo and C.D. Kirkwood, unpublished data, 1996; U59108), and NCDV [Powell et al., 1988] (X06806) have been previously described.

such as simian serotype G3 strain SA11 and bovine serotype G6 strains UK, and NCDV as well as the genetic relationship between porcine strain YM and typical subgroup II HRVs.

Phylogenetic analysis of the corresponding nt sequences gave a pattern that was essentially identical [i.e., three distinct genetic groups represented by prototype strains RV-5 (genetic group I), Wa (genetic group II), and RRV (genetic group III)]. However, as was suggested by the nt divergence data shown in Table III, the corresponding nt sequences of animal rotaviruses such as SA11 and YM were somewhat more divergent from HRV isolates of the corresponding genetic group compared to the analysis performed with deduced aa sequences (not shown).

DISCUSSION

Using RT-PCR amplification of fragments of the NSP4 gene followed by nt sequencing of the PCR products, we obtained NSP4 sequence data from 16 HRVs from six countries; seven different G and P types were included in this collection. These data were compared

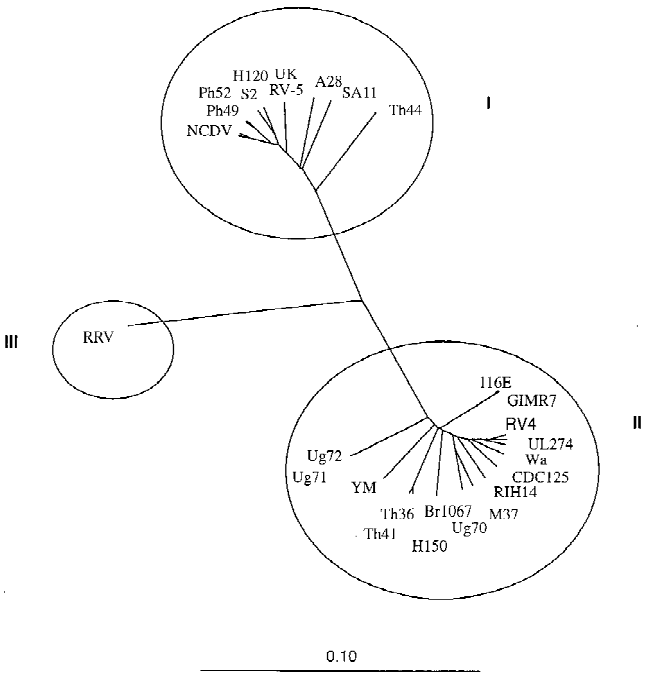

Fig. 2. Phylogram of NSP4 peptides. The phylogenetic tree was generated from a multiple sequence file, using the Protdist and Kitsch modules of the Phylip 3.5 program to calculate a distance matrix and draw the tree [Devereux et al., 1984; Felsenstein, 1989]. Genetic groups I, II, and III are circled.

to six previously reported NSP4 sequences (strains Wa, A28, UK, NCDV, YM, and SA11) and five unpublished NSP4 sequences (strains RRV, RV-5, S2, M37, and RV4), including both human and animal rotaviruses [Baybutt and McCrae, 1984; Both et al., 1983; Okada et al., 1984; Ward et al., 1985; Ballard et al., 1992; Powell et al., 1988; Lopez and Arias, 1993] (E.A. Palombo and C.D. Kirkwood, unpublished data, 1996; B. Tang and H.B. Greenberg, 1996). Based on the amount of sequence divergence between strains, three distinct genetic groups could be recognized, two of which contained representatives of common HRV serotypes, and one containing the vaccine strain RRV alone. Generally, strains within a group were highly conserved (<10% divergence) at the nt and aa levels with regard to each other but were distantly related to strains in the other groups. For all HRV strains, these relationships followed the same patterns defined by RNA-RNA hybridization in that the strains expected to belong to the Wa genogroup based on published information [Nakagomi et al., 1989; Nakagomi and Nakagomi, 1989; Flores et al., 1982; i.e., type G1, G3, G4, and G9 strains with subgroup II specificity) were members of a single, larger NSP4 genetic group II and those expected to fall into the DS-1 genogroup (type G2 with subgroup I specificity) formed a second, smaller NSP4 genetic group I. In addition, two HRV isolates, a Brazilian isolate (Br1067) and an isolate from an asymptomatic neonate in India (116E), were closely related to other HRV strains within NSP4 genetic group II. Representatives of both these strain types (including 116E) that have been genogrouped by RNA-RNA hybridization were apparent reassortants, deriving most genes from the Wa genogroup and one to a few genes from animal rotaviruses [Leite et al., 1996; Das et al., 1993]. The NSP4 gene of RRV was distinct from all other strains and formed genetic group III.

Unexpectedly, the NSP4 genes of some animal rotaviruses (UK, NCDV, SA11, YM) and a G serotype 10 HRV strain recovered from a child with severe combined immunodeficiency syndrome [Baybutt and McCrae, 1984; Both et al., 1983; Ward et al., 1985; Ballard et al., 1992; Powell et al., 1988; Lopez and Arias, 1993] could be classified in one of these NSP4 genetic groups. This observation suggests a strong evolutionary relationship between the NSP4 genes of human and animal rotaviruses, which for some strains might also be explained in terms of genogroup relationships [e.g., strain YM NSP4 gene is 88% and 95% identical at the nt and aa levels, respectively, to the Wa NSP4 gene and shows significant relatedness to Wa genogroup strains by RNA-RNA hybridization (Kojima et al., 1996)]. Furthermore, homology between the G5 porcine strain OSU and the Wa genogroup has also been shown by RNA-RNA hybridization [Nakagomi and Nakagomi, 1991]. Although the simian strain SA11 is quite distinct from the HRV Wa and DS-1 genogroups, at least one hybrid band that migrates more slowly than bona fide gene 10 shows homology to the DS-1 genogroup using an SA11 hybridization probe [Nakagomi and Nakagomi, 1991]. Thus, even for SA11, our results might be explained in terms of genogroup relationships, although it should be noted that, in the genogrouping studies cited here, the origin of gene 10 could not be identified with certainty. However, because SA11 is virtually distinct from HRV genogroups, excluding gene 10, it could be speculated that NSP4 genes diverged either by mutation or by reassortment prior to the divergence of the remainder of the rotavirus genome segments. The relationship between porcine rotavirus and the human Wa genogroup supports this hypothesis; the porcine YM NSP4 as well as several of its other gene segments are closely related to the Wa genogroup, whereas the remaining genes are more divergent [Kojima et al., 1996]. The mechanism behind the apparent evolution of the NSP4 gene independently from the evolution of genogroups is unknown but could be related to its unique role in viral assembly.

Although most of the sequences within NSP4 genetic groups are highly conserved, some strains are more divergent (e.g., Ug71 and Ug72, genetic group II strains isolated from Uganda and Th44, a genetic group I isolate from Thailand), and it is possible that additional genetic groups will be detected as more strains are characterized. For example, the NSP4 gene of strains representing the HRV AU-1 genogroup having subgroup I specificity and a very close genetic relationships to feline rotaviruses [Kitaoka et al., 1987; Nakagomi et al., 1989], as well as other feline-canine-like strains identified in Brazilian and Israeli infants [Nakagomi et al., 1989; Timenetsky et al., 1994; Silber-

stein et al., 1995], have not been characterized. As was observed for intergroup variation, much of the intragroup divergence occurred in two highly variable regions of NSP4, including the carboxy terminal 23% of the toxic peptide (aa 131–135) and adjacent aa residues in the carboxy terminus of the NSP4 protein (aa 131–159).

Insofar as the NSP4 toxic peptide (aa 114–135) appears to be the diarrheagenic region of NSP4, at least in the mouse model, it might be expected that the aa sequence of this peptide (or at least the key aa required for its biological activity) would be conserved between rotavirus strains that cause diarrhea. Consistent with this possibility, the amino terminus of the toxic peptide of all strains analyzed was highly conserved. Tyrosine 131 has been shown to be critical for the diarrheagenic activity of the toxic peptide [Ball et al., 1996]. For the strains analyzed here and for previously reported NSP4 sequences, tyrosine 131 is conserved only within genetic group I, whereas most members of genetic groups II and III possessed histidine at this position. Exceptions to these findings at aa position 131 were in genetic group II (116E and GIMR7), both of which possessed tyrosine, and in genetic group I (Ph49), which possessed histidine. This suggests that, if NSP4 is a HRV virulence factor, then strains possessing histidine at position 131 are also capable of inducing diarrhea. Furthermore, a recent report suggests that substitutions in the carboxy terminus of the toxic peptide have a profound effect on its ability to cause diarrhea in a mouse model [Zhang et al., 1996]. The finding of a high degree of variation in this region between different NSP4 genetic groups could, if NSP4 is an important virulence gene, explain some of the differences in the clinically observed outcomes of natural rotavirus infections. For example, serotype G2 strains (expected to belong to NSP4 genetic group I described here) have been shown to be more virulent than other HRV strains in some studies [Bern et al., 1992; Timenetsky et al., 1996].

The NSP4 protein has also been shown to have membrane-destabilizing [Tian et al., 1996] and virus-binding activities [Taylor et al., 1996] that might be involved in rotavirus assembly in the endoplasmic reticulum of infected cells. The toxic peptide was shown to play an integral role in the membrane-destabilizing activity (MDA) of NSP4, and, more specifically, tyrosine 131 might also be a crucial site for this activity, in that iodination of this aa abolished its MDA [Tian et al., 1996]. In this study, we have shown that tyrosine 131 varies between genetic groups, suggesting that other aa at this site (e.g., histidine) presumably can promote the MDA associated with NSP4. Furthermore, we have found that at least some of the carboxy-terminal 20 aa proposed to be important for NSP4 binding to immature virus during assembly also vary between NSP4 genetic groups. Thus, the results presented here might further contribute to our understanding of virus assembly.

In addition, it is possible that comparative sequence analysis of the toxic peptide region of NSP4 genes of classic asymptomatic neonatal "nursery" strains will aid our understanding of their apparent attenuation. Thus, although the NSP4 sequence of Ugandan strain Ug71 (detected in a 9-day-old hospital-born neonate without diarrhea) is in the same genetic group (II) as common childhood diarrhea strains of serotypes G1, G3, or G4, this strain was more divergent than most other members of this group, differing in an aa sequence that included two substitutions just downstream of the toxic peptide region at positions 137 (lysine) and 138 (serine). A recent report from Australia described specific conserved aa differences at position 135 (valine or isoleucine) in the NSP4 genes of P[6]G3 strains that cause symptomatic (valine) compared to asymptomatic (isoleucine) neonatal infections [Kirkwood et al., 1996]. Two strains from Uganda examined in this study (neonatal strain Ug71, and Ug72, a community strain obtained from a 5-month-old infant with acute diarrhea) each contained isoleucine at position 135. However, a limitation of this study is that we have not specifically addressed the question of whether P[6] strains from asymptomatic neonates differ significantly with respect to their NSP4 genes compared to P[6] strains isolated from older symptomatic children. This issue should be addressed in a separate study.

The RRV vaccine strain was highly divergent (including differences in the carboxy terminus of the toxic peptide region) from all other strains examined and formed a third genetic group. This strain has little homology to either the Wa or the DS-1 genogroups in RNA-RNA hybridization analyses [Nakagomi and Nakagomi, 1991]. In contrast, the bovine strain UK (related to vaccine strain WC-3) was highly homologous to RV-5 and belonged to genetic group I. The implications of these findings for the use of RRV and WC-3 as rotavirus vaccines are at present unknown.

A limitation of this study relates to the culture-adapted strains used (GIMR7, UL274, CDC125, RIH14, and 116E), for which passage might possibly have selected changes in the NSP4 gene sequence. However, this possibility is unlikely to alter the major conclusions of this study; most NSP4 aa sequences are conserved within this genetic group, among both culture-adapted and fecal strains.

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